AWARD NUMBER: W81XWH-11-1-0583

TITLE: "Mitochondrial-Based Treatments that Prevent Post-Traumatic Osteoarthritis in a Translational Large Animal Intraarticular Fracture Survival Model"

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REPORT DATE: September 2015

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
September 2015	Annual	1 Sep 2014 – 31 Aug 2015

4. TITLE AND SUBTITLE
"Mitochondrial-Based Treatments that Prevent Post-Traumatic Osteoarthritis in a Translational Large Animal Intraarticular Fracture Survival Model"

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-11-1-0583

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Iowa
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Iowa City IA 52242-1316

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The purpose of this research is to investigate a novel therapeutic approach to prevent PTOA by treating mitochondrial dysfunction in chondrocytes resulting from an intraarticular injury. To date, we have shown that all of the drugs targeting mitochondrial mechanotransduction and oxidant production that we have tested are chondroprotective to some degree. We are currently awaiting pig model sacrifices upon completion of the 6- and 12-month time points.

15. SUBJECT TERMS
Post-traumatic osteoarthritis, oxidative stress, mitochondria, animal model

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	11	19b. TELEPHONE NUMBER *(include area code)*

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Introduction

Experimental Overview

The purpose of this research is to investigate a novel therapeutic approach to prevent PTOA by treating mitochondrial dysfunction in chondrocytes resulting from a intraarticular injury. We have shown that scavenging excessive injury-related mitochondrial oxidants, or preventing their excessive formation after a severe impact injury to cartilage in a tissue-level model prevented chondrocyte death in a bovine explanted tibial cartilage [1-3]. Subsequently, we demonstrated that oxidant production is strain –dependent and that physiologic levels of mitochondrial oxidants, generated in tissue samples subjected to normal loads, were important promoters of chondrocyte glycolytic ATP synthesis[4,5]. These findings are the basis of the treatment strategies pursued in this *in vivo* investigation.

Keywords
post-traumatic osteoarthritis, oxidative stress, mitochondria, animal model

Summary of Progress

The live animal experiments were completed in August 2015. The last pigs included additional 6 month follow-ups, and for the first time, 12-month follow-ups. The effects of NAC were assessed at6 months. We were delighted to discover that, with 2 exceptions, all amobarbital and NAC treated joints (n = 8/group) showed little if any signs of progressive OA. The exceptional cases showed excessive wear on the medial talus. This proved to be due to misplacement of fixation screws, which rubbed on the medial talus. Sham operated animals also showed with no fracture but with

An EWOF request was submitted on September 2 2015 to extend the original end date of 9/30/2016, a period of 12 months beyond the original end date. No additional funds are being requested. Processing for paraffin histology is complete and 75% of the specimens have been sectioned, stained with safranin-O/fast green, and scanned at 20X. Sections will be scored for OA using our automated Mankin system. Processing, sectioning, and staining of synovia is also complete. These will be scored for monocyte infiltration using customized Visiomorph-based computer software. Synovial fluid samples for cytokine assays were collected and assays have been started. Seahorse assays for respiratory activity are complete. A manuscript reporting these data has been initiated in a format compatible with Nature Medicine. Because this work reveals a molecular mechanism underlying PTOA we are optimistic that our submission will be seriously considered by this highly rated journal.

The three Specific Aims, the associated Statement of Work, and progress to date are outlined below. The following Statement of Work is as was approved during PI transfer, with official modification approved/signed on December 17, 2013 by Dana Herndon:

Specific Aim 1. Measure changes in chondrocyte ATP production, , chondrocyte viability, and PTOA in a survival rabbit model of cartilage injury treated with electron transport complex I inhibition and ROS scavenging.

Task 1: Surgical injury to the rabbit medial femoral condyle (Months 1-36)

A. Experimental groups (N = 6/group; 6 animals sacrificed at 7 days, 6 animals treated with best treatment sacrificed at 42 days, and at 180 days post-op; 24 rabbits total)

1. Amobarbital (complex I inhibitor)
2. N-Acetylcysteine (ROS scavenger)

B. Control groups (N = 18/group; 6 animals sacrificed at each 7 days, 42 days, and 180 days post op; 42 rabbits total)

1. Impact control (rabbits receive injury but no treatment)

Mitochondrial Based Treatments that Prevent Post-Traumatic Osteoarthritis in a Translational Large Animal Intraarticular Fracture Survival Model

Year 4 Technical Progress Report: W81XWH-11-1-0583 (award #14793800).

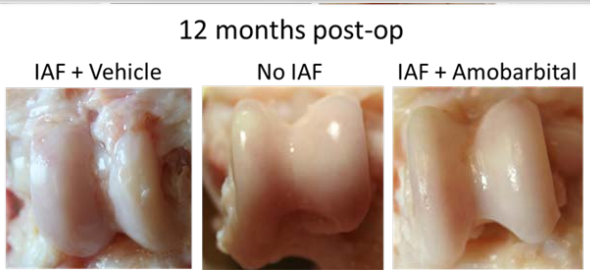
PI: James A. Martin **Org:** The University of Iowa **Award Amount:** $2,559,912 (original), $2,559,259 (modified, incorporated by reference only according to award documents signed 17 Dec 2013)

Study/Product Aim(s)

- Aim 1: Measure the effects of free radical scavengers and inhibitors of electron transport on chondrocyte viability and metabolism and PTOA in a survival rabbit model .
- Aim 2: Determine the effects of compounds that dissolve filamentous actin and microtubulin in the rabbit model.
- Aim 3: Determine the efficacy of mitochondrial-based treatments on preventing PTOA in a large animal IAF survival model.

Approach

Yucatan minipigs with hock joint fractures were treated with amobarbital or NAC by intra-articular injection at time 0 and 1 week post-op and were allowed free activity for 6 or 12 months before sacrifice. Fresh cartilage was harvested for analysis of respiratory activity and the remaining cartilage and bone were processed for paraffin histology. Synovial fluid and synovium were also collected for cytokine assays and for monocyte counting respectively.



Representative tali from fractured hock joints at 12 months post-injury (n =8/group). Without treatment (left panel) cartilage degeneration was readily apparent (arrows) whereas the tali of pigs treated by intra-articular injections of amobarbital at time 0 and 1 week post- fracture (right panel) looked similar to uninjured contralateral tali (middle panel).

Accomplishment: NAC and amobarbital were similarly effective at 6 months and amobarbital prevented PTOA for up to 12 months post fracture.

Timeline and Cost

Activities / CY	12	13	14	15
(Aim 1)	Short-term tests (rabbit)			
(Aim 2)			Long-term tests (rabbit)	
(Aim 3)				Porcine tests
Estimated Budget ($K)	**$380,245**	**$521,666**	**$819,756**	**$837,592**

Updated: 2 October 2014

Goals/Milestones

CY12-13 Goals – Specific Aim 1
☑ Obtain results for short-term rabbit (and pig) models
CY13-14 Goals – Specific Aim 2
☑ Begin histological processing on long-term rabbit models using Amobarbital and NAC in hydrogel, single injection
CY14-15 Goal – Specific Aim 3
☑ Begin long-term pig IAF model first doing 6-month sham and injured control animals, then treatment animals
☑ Complete surgery on all IAF pig model animals
CY15 Goal – Wrap Up Project (80% complete)
☐ Finish histology on all remaining long-term rabbit and all IAF pig models, data analysis, and publish results

Budget Expenditure to Date (through August 31, 2015)
Projected Expenditure: $1,721,667
Actual Expenditure: $1,613,147

2. Sham control (rabbits receive surgery but no injury or treatment)
3. Normal control (no surgery or treatment; only 6 rabbits in this group)

Task 2: Confocal, Biochemical, and Histologic Analyses (Months 1-36)

A. Confocal imaging

1. Chondrocyte viability determined by Calcein-AM staining. Live cell density will be calculated.
2. Images analyzed with in-house software for cell counts

B. Biochemical Analysis

1. ATP production determined with assay of cartilage directly

C. Histologic Analysis

1. Safranin O staining to determine degeneration scores (HHGS scale of Mankin)
2. Immunohistology to determine MMP-3, MMP-13

Specific Aim 2. Measure changes in chondrocyte ATP production, chondrocyte viability, and PTOA in a survival rabbit model of cartilage injury treated with compounds that dissolve filamentous actin and microtubulin.

Task 1: Surgical injury to the rabbit medial femoral condyle (Months 1-36)

A. Experimental Groups (N = 6/group; 6 animals sacrificed at 7 days, 6 animals treated with the best treatment sacrificed at 42 days, and at 180 days post-op; 24 rabbits total)

1. Cytochalasin B
2. Nocodazole

B. Control Groups: same animals used in Specific Aim 1 for Impact Control, Sham Control, and Uninjured Control

Task 2: Confocal, Biochemical, and Histologic Analyses (Months 1-36). This is identical to Specific Aim 1.

Work to Date Specific Aims 1 and 2

A total of 122 Rabbit surgeries were completed as of February 2014. All histology is complete and image analysis is nearly complete. It was decided to publish the 1 week and 8 week data together and that manuscript is in preparation.

Specific Aim 3: Determine the efficacy of treatments that prevent ROS overproduction, scavenge ROS, or dissolve the cytoskeleton in mitochondria on preventing PTOA in a large animal IAF survival model.

Task 1: Surgical creation of a physiologic realistic intraarticular fracture in the minipig specimens (Months 25 – 48; 48 pigs total)

A. Experimental Groups (12 animals/group; six animals sacrificed at 6 months and six animals sacrificed at 12 months; 24 pigs total)

1. Treatment one
2. Treatment two

B. Additional Control Groups (Months 25 – 48)

1. Injured Control: 12 animals (six sacrificed at 6 months and six sacrificed at 12 months after surgery)
2. Sham Control: (No impact injury, but hardware placement): 12 animals (six sacrificed at 6 months and six sacrificed at 12 months after surgery)

Work to Date on Specific Aim 3

The last of the 6 month and 12 month survival animals were euthanized in August 2015. Joint surfaces were photographed (Figure 1), and then processed for paraffin histology. The latter involves fixation, decalcification, and dehydration, which takes up to 6 weeks with specimens of this size. Sagittal sections through loaded areas of the talus and tibia Safranin-O-stained are being scanned in preparation for quantitative image analysis to assess cartilage degeneration. Representative samples are shown below (Figure 2). Synovial fluid assays (cytokine microarrays) are under way. Joint capsules were paraffin-embedded, sectioned, and stained with H&E. These are being scanned and will be scored for monocyte infiltration. Seahorse assays of chondrocyte respiratory activity were completed (Figure 3).

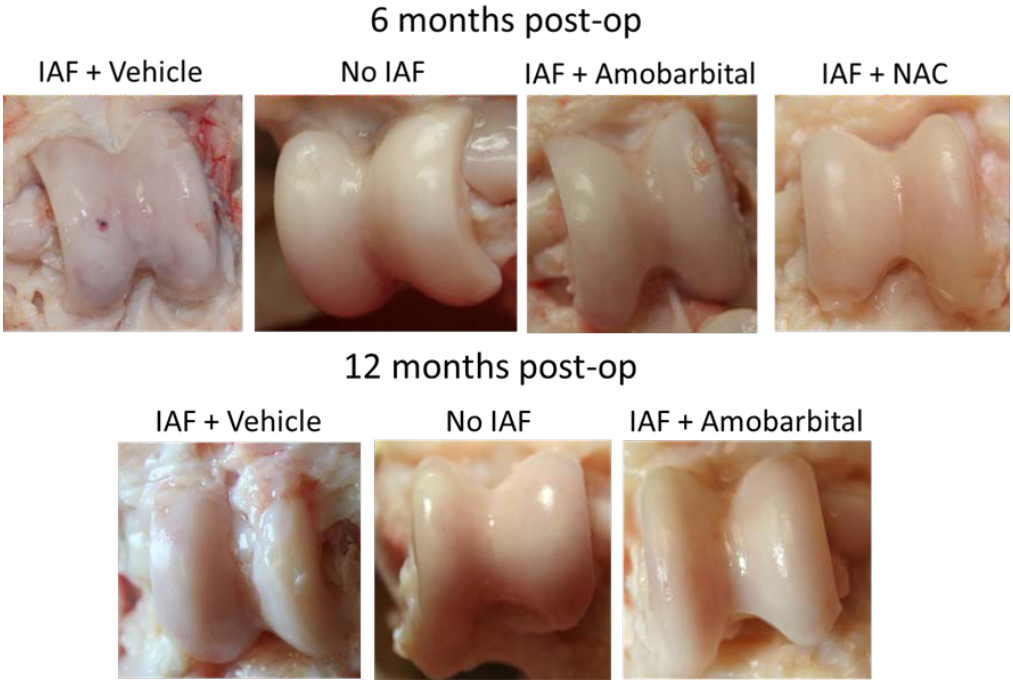


Figure 1. Chondroprotective effects of amobarbital in a porcine IAF model. Freshly-harvested tali from the hock joints of pigs were photographed soon after euthanasia at 6 months and 12 months post-op. Multiple cartilage lesions are apparent in the fractured, untreated specimen IAF +Vehicle. In contrast, the surfaces of the tali from fractured joints treated with 0.5 ml of 2.5 mM amobarbital or 10 mM NAC were relatively smooth, and resembled the intact contralateral joints more than the untreated fractured joints.

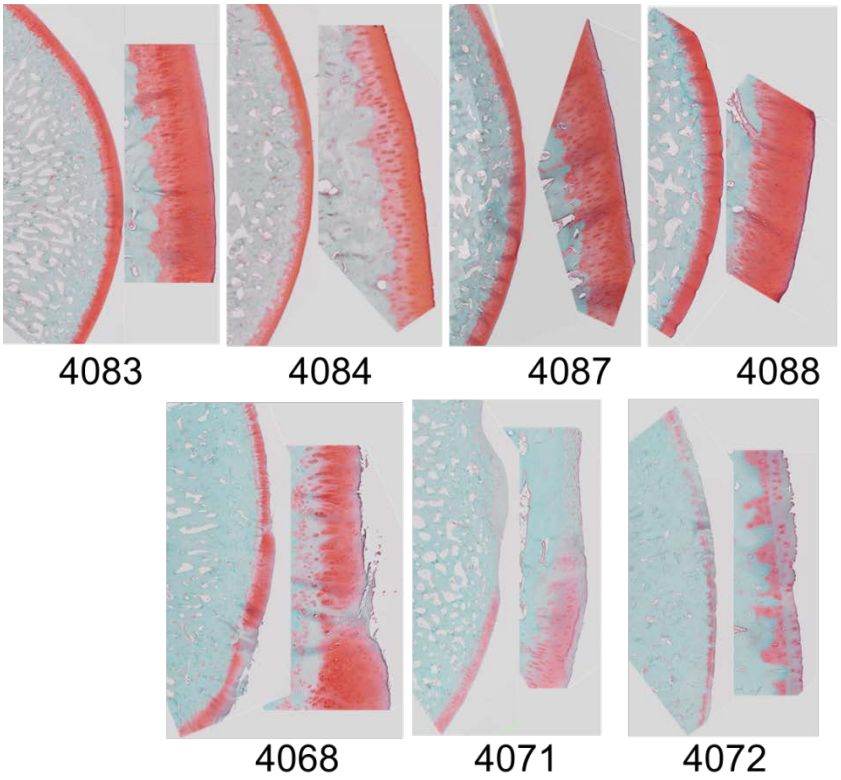


Figure 2. Safranin-O stained sections of tali from amobarbital-treated hocks and from untreated (vehicle only) hocks. Each frame shows the entire cross section and zoomed-in insets. The staining pattern for the amobarbital-treated group (4083, 4087, and 4088) is essentially normal, with no visible surface damage, high concentrations of proteoglycan through the depth, and normal cellularity. In the untreated specimens (4068, 4071, 4072) there was varying degrees of cartilage degeneration including substantial loss of proteoglycans, cartilage erosion, hypocellularity, and tidemark disruption. The remaining samples (n = 8/group) are still being processed.

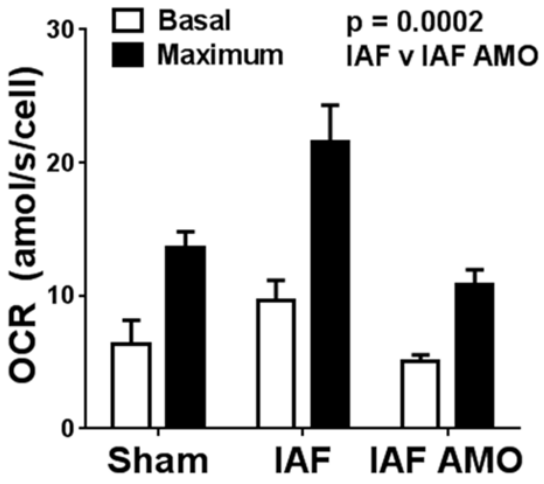


Figure 3. Amobarbital prevents osteoarthritis-related increases in respiration rates. A Seahorse Extracellular Flux analyzer was used to measure per-cell oxygen consumption rates (OCR) in chondrocytes. Basal and maximum OCR (OCR in the absence or presence of a depolarizing agent) was measured in talar chondrocytes isolated at 6 months post-op from sham-operated controls, from fractured joints that were not treated (IAF), and from fractured joints treated with amobarbital at time 0 and 1 week as described above (IAF AMO). Normal OCR values were seen in the sham group, which exhibited little if any OA. Levels increased above normal in the IAF group where OA was evident, but remained close to normal in the AMO group, which was devoid of OA changes. Columns and error bars represent means and standard errors based on 8 samples per group.

Key Research Accomplishments

1. Showed that acute NAC was as effective as amobarbital in preventing PTOA at the 6 month follow-up.

2. Showed that amobarbital prevented PTOA for up to 12 months post-fracture.

3. Demonstrated that NAC and amobarbital prevented OA-like increases in chondrocyte respiration.

Related Work

The original developmental study generated hock joints specimens harvested 1 week after fracture. According to our theory of PTOA, we expected to see signs of oxidative stress in cartilage at this time point. A primary cellular response to oxidative stress can be to activate the transcription factor NRF2. Oxidation of protein thiol residues results in stabilization of NRF2 and promotes its translocation to the nucleus, where it up-regulates a number of antioxidant-related genes. Immunohistochemical analysis showed that while basal NRF2 levels in chondrocytes were very low, the protein was abundant in cells at 1 week post-IAF (Figure 4). These results support the hypothesis that chondrocytes responded to the oxidative stress indicated by increases in GSSG:GSH ratio (Figure 5). NAC given immediately post-IAF almost entirely blocked IAF-induced NRF2 activation, and increased GSSG:GSH ratio, suggesting it prevented oxidative stress.

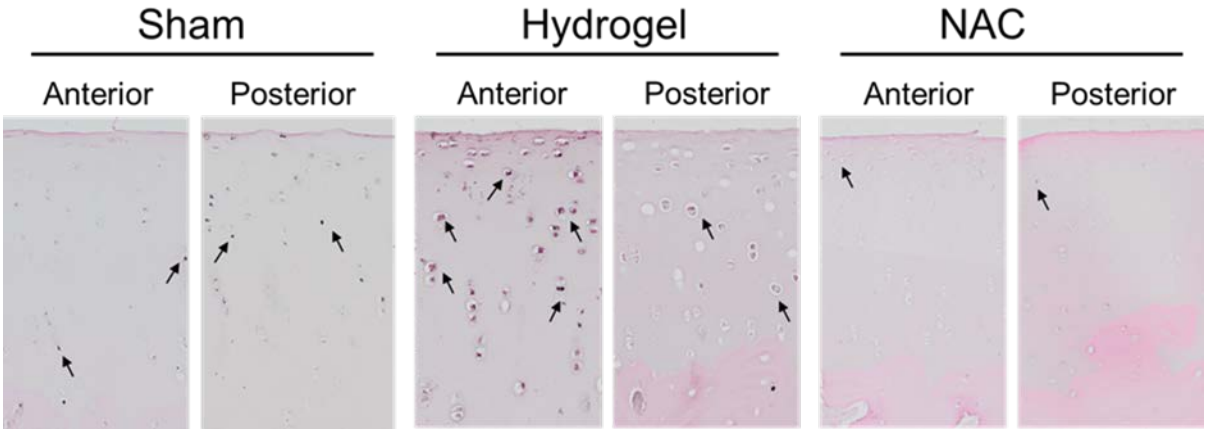


Figure 4. Immunohistochemical staining of talar cartilage for NRF2. Cartilage was harvested at 1 week post-op from sham-operated hocks, fractured hocks without treatment (hydrogel vehicle only), and fractured hocks treated with 10 mM NAC immediately after IAF. De-paraffinized sections were stained overnight with anti-NRF2 antibody and counterstained with eosin. While a few positives were seen in the sham operated samples, staining was much more intense and widespread in fractured hydrogel-injected hocks, indicating a cellular response to oxidative stress. Little if any staining was observed in the NAC treatment group, indicating that cells were maintaining redox balance

Conclusions
With much of the data collected we can conclude that it is possible to forestall the development of PTOA with just one or two treatments during the first week post-fracture. The outstanding chondroprotective effects of acute intra-articular injection of either amobarbital or NAC in the IAF model leave little doubt that the disease mechanism we have proposed is indeed operative in PTOA. There was no evidence of toxicity.

Publications, Abstracts, and Presentations
Coleman MC, Ramakrishnan PS, Brouillette MJ, Martin JA. Injurious Loading of Articular Cartilage Compromises Chondrocyte Respiratory Function. Arthritis Rheumatol. 2015 Oct 16. doi: 10.1002/art.39460. [Epub ahead of print]

Coleman et al. Administration of N-Acetylcysteine Prevents Progression of Post-Traumatic Osteoarthritis in a Large Animal Model of Intraarticular Fracture. Accepted for podium presentation by The Society for Redox Biology and Medicine for presentation in 2015 with a $500 travel award.

Coleman MC, Goetz JE, Peterson EE, Willey M , Bergh MS, Fredericks DC, McKinley TO, Martin JA. N-Acetylcysteine Prevents Acute Chondrocyte Injury and Dysfunction Associated with Osteoarthritic Progression after Intraarticular Fracture. Military Health System Research Symposium 2015

Inventions, Patents and Licenses
Provisional patent covering intra-articular delivery of amobarbital to prevent PTOA (A PREVENTIVE THERAPY FOR POST-TRAUMATIC OSTEOARTHRITIS) was filed on behalf of Dr Martin and colleagues by the University of Iowa Research Foundation in August 2015. The application states the following: “This invention was made with government support under grant number W81XWH-11-1-0583 awarded by the Department of Defense. The government has certain rights in the invention”.

Reportable Outcomes.
A manuscript based on the findings will be submitted for publication in Nature Medicine. The data are sufficient to begin the process of applying to the FDA for approval for use in humans. With intellectual property protection in hand we plan to form a company to seek private investors to fund clinical trials.

Other Achievements
1. Dr. Marc Brouillette graduated with a Ph.D. in August 2015.

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